B&P File No. 571-914/PP

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UNITED STATES

Title: METHOD OF IMMOBILIZING MEMBRANE-ASSOCIATED MOLECULES

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FIELD OF THE INVENTION

The present invention relates to methods for the immobilization of membrane-associated molecules, including membrane-associated biomolecules, to composites prepared by such methods and to the use of these composites, in particular for high-throughput drug screening, multianalyte biosensing or bioaffinity chromatography.

BACKGROUND TO THE INVENTION

Immobilization of natural cellular receptors, which are mainly membrane associated proteins, is receiving substantial attention in the areas of research, clinical and environmental analysis, and in drug development.[1,2,3,4,5,6,7,8,9,10,11] This is a result of increasing demand for robust and portable devices for medical, environmental and bioprocess monitoring. Just as the immobilization of biomolecules such as polynucleotides in the microarray platform has revolutionized the area of genomics, the immobilization of proteins will provide the same advantage to proteomics.[12,13,14,15] Furthermore, immobilization of proteins provides additional advantages in the area of small molecule drug-screening using both microarray[16] and chromatographic platforms.[17] Thusfar, protein immobilization has focused mainly on soluble proteins, which are usually more robust than their membrane-bound counterparts. The major problems limiting the development of new sensors and high-throughput screening technologies that utilize these cellular receptors arise due to the inherently low stability of such receptors and the difficulties associated with transducing receptor-ligand binding events into measurable signals. However, membrane-bound receptors are particularly attractive targets for the development of new diagnostic devices and for discovery of new therapeutic treatments and drugs. Therefore, robust and facile immobilization techniques are needed to accommodate the sensitive supramolecular assemblies of proteins, and other membrane-associated molecules, within lipid bilayers.

A number of strategies have been reported for the immobilization of bilayer lipid membranes (BLMs) and membrane-bound receptors, including supporting of BLMs on the pores of filter paper,[18] covalent attachment of monolayer or bilayer lipid

membranes to surfaces,[1,4,5,19] tethering of phospholipid liposomes to a surface by deposition,[20] covalent attachment[21] or via avidin-biotin linkages,[22] and entrapment of BLMs into polymer multilayers to provide a semihydrated internal surface to allow incorporation of bulkier membrane receptors and proteins.[5] However, such immobilization methods have been observed to reduce the natural dynamic motions of the bilayers, and lead to unstable immobilized structures.[23,24,25,26,27,28,29,30] Problems can also arise due to the coupling of the lipid bilayer to the solid support, which can produce an unstable structure with a lifetime that is too short for functional purposes. Furthermore, the structure of intrinsic membrane-proteins relies on hydrophobic interactions internal to the lipid bilayer, as well as hydrophilic interactions on either side of the lipid membrane.[5] Very often with conventional supported BLMs, what would be considered the hydrophilic interior surface for the membrane protein is replaced by the solid substrate.[5,31] This situation results in destabilization of the membrane protein with a concomitant loss in activity, or in the worst-case scenario complete loss of activity due to full denaturation of the protein. These issues have been partially addressed by covalent attachment of a lipid monolayer to a solid substrate, which alleviates membrane dissociation; however, this method does not address the second issue mentioned, and furthermore decreases the natural dynamic behavior of the bilayer.[4,5]

An emerging method for the immobilization of biological species is their entrapment within inorganic matrixes formed by the sol-gel processing method.[32,33] This method involves formation of a colloidal sol solution owing to hydrolysis of a precursor such as tetraethyl orthosilicate (TEOS). A buffered solution containing the biomolecule of interest is then added to the sol to initiate rapid polycondensation of the silane. Following polycondensation a hydrated gel is produced that immobilizes the biological element without the need for a covalent tether.

Entrapment of soluble proteins in sol-gel derived silicate has proven to be an advantageous method for maintaining protein dynamics and activity over periods of months or more. However, the sol-gel method has found much less use for the immobilization of membrane-bound proteins. Indeed, only a few reports exist describing the immobilization of liposomes[34,35,36,37,38] or whole cells[39,40,41,42,43,44] into inorganic silica matrixes formed by the sol-gel method, and only a single membrane-

associated protein, the photo-active receptor bacteriorhodopsin (bR), has been successfully entrapped in sol-gel derived silica.[45,46,47,48] However, even these reports describe the entrapment of bR that was associated with only its intrinsic lipids, rather than bR that was reconstituted into a phospholipid bilayer membrane. Furthermore, the activity of entrapped bR was assessed by monitoring the decay from a photoactivated conformational intermediate referred to as the M-state, and did not directly measure ligand binding or ion channeling by entrapped bR. Limitations in the ability to monitor the ion channel activity of membrane proteins entrapped in sol-gel derived silicate may have arisen due to the selection of sol-gel precursor. During the hydrolysis of the silane precursors tetraethylorthosilicate (TEOS) of tetramethyl orthosilicate (TMOS) ethanol or methanol are produced. These byproducts will readily dissolve or destabilize existing bilayer structures.[33] Without a stable liposome, ion flux or membrane potential cannot be developed and therefore cannot be measured.

For commercial applications, there remains a need for a method of entrapping membrane-associated molecules as liposome assemblies that maintain the stability of both the membrane associated molecule and the bilayer lipid membrane.

SUMMARY OF THE INVENTION

A new method for the immobilization of membrane-associated proteins or ionophore-liposome assemblies has been developed. This method is based on the immobilization of a reconstituted molecule-liposome assembly within a sol-gel-derived matrix that is prepared from protein- and membrane-compatible precursors, such as diglycerylsilane (DGS) and sodium silicate. Specifically, the ion channel proteins, gramicidin A (gA) and nicotinic acetylcholine receptor (AChR), as well as the Ca(II) ionophore ionomycin, embedded within the membranes of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) liposomes or more complex liposomes, were immobilized into DGS- or sodium silicate-derived sol-gel materials and it was shown that, upon immobilization, the gramicidin, nAChR or ionomycin remained embedded in the phospholipid membrane and their ligand-binding ability and/or transmembrane ion flux activity was retained. In addition, gramicidin remained sensitive to the concentration of ions across the membrane and selective to passage of monovalent cations through the peptide channel, ionomycin retained the ability to transport Ca(II)

across the membrane, while nAChR retained its ability to transport Ca(II) across the membrane in a ligand-gated fashion based on its interaction with agonists. Furthermore, following immobilization of gramicidin, the ability of divalent cations to block ion flux through the channel was also retained, while nAChR retained the ability to be inhibited by known antagonists, which block the ion channel, indicating that modulation of membrane-channel proteins is possible following entrapment in sol-gel derived silica.

Accordingly, the present invention relates to a method of immobilizing membrane-associated molecules in silica matrixes comprising combining a liposome-assembly comprising the membrane-associated molecule, with a protein- and membrane-compatible sol-gel precursor under conditions which allow a gel to form.

The present invention further relates to protein- and membrane-compatible sol-gels with a liposome-membrane associated molecule assembly immobilized therein.

Further included within the scope of the present invention are methods for the detection of modulators of membrane-associated molecules comprising:

(a) exposing a liposome assembly comprising the membrane-associated molecule, said assembly being immobilized in a protein- and membrane-compatible sol-gel, to one or more test substances; and

(b) detecting a change in one or more characteristics of the membrane-associated molecule.

In embodiments of the invention the protein- and membrane-compatible sol-gel is prepared using a method as described herein. In further embodiments of the invention, a change in the one or more characteristics of the membrane-associated molecule in the presence of the one or more test substances compared to a control indicates that the one or more test substances are modulators of the membrane-associated molecule.

The methods of entrapment and for detecting modulators of membrane-associated molecules of the present invention provide a general method for analyzing these molecules and their inhibitors, agonists and/or antagonists. The ability to immobilize membrane-associated molecules will allow development of bioaffinity

chromatography or microarray technologies that will be useful for high throughput screening of potential inhibitors or effectors.

Additionally, a novel procedure amenable to the sol-gel method of entrapment has been developed to monitor ion flux through an entrapped membrane-associated molecule. In this method, the fluorescence indicator used to detect the development of a potential (due to ion flux) across the lipid membrane was located on the inside of the liposome assembly only. Literature methods describe the use of fluorescent indicators in the both the internal and external solution. Problems caused by interactions of the indicator molecule with the anionic surface of the silica can arise when the indicator molecules are in the external solution. Such problems are avoided when the indicator molecules are located within the interior of the liposome assembly since, in this location, these molecules are not able to interact with the silica surfaces. Accordingly, the present invention further relates to an improved method for preparing a sol gel immobilized liposome assembly comprising a membrane associated molecule, wherein the membrane-associated molecule is an ion-channel molecule, comprising:

(a) obtaining a solution of the liposome assembly having an indicator molecule located on the interior of the assembly;

(b) removing the indicator molecule from solution external to the liposome assembly; and

(c) combining the liposome assembly solution with a silica precursor solution under conditions which allow a gel to form.

The present invention also relates to an improved method for the detection of membrane potentials in a sol-gel immobilized liposome assembly comprising a membrane-associated molecule, wherein the membrane-associated molecule is an ion-channel molecule, comprising:

(a) obtaining a solution of the liposome assembly having an indicator molecule located on the interior of the assembly;

(b) removing the indicator molecule from solution external to the liposome assembly;

(c) combining the liposome assembly solution with a silica precursor solution under conditions which allow a gel to form;

(d) contacting the gel with the ion and optionally a test substance; and

(e) detecting a change in the indicator molecule upon transmembrane ion flux.

The present invention also includes kits, biosensors, microarrays, chromatographic and bioaffinity columns comprising the silica matrixes comprising a liposome-protein assembly prepared as described herein.

Yet another aspect of the present invention provides a method of conducting a target discovery business comprising:

(a) providing one or more assay systems for identifying test substances by their ability to effect one or more membrane-associated molecules based systems, said assay systems using a method of the invention;

(b) (optionally) conducting therapeutic profiling of the test substances identified in step (a) for efficacy and toxicity in animals; and

(c) licensing, to a third party, the rights for further drug development and/or sales or test substances identified in step (a), or analogs thereof.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1 shows tryptophan emission spectra of gramicidin A before and after reconstitution into phospholipid vesicles comprised of DOPC, both in solution and after entrapment into DGS derived silica.

Figure 2 is a schematic of the response of safranine O to the development of a membrane potential caused by an influx of potassium ions under various conditions. (a) Conventional method with safranine O located on the exterior of the liposome and (b) the "inverted" method, employing safranine O on the interior of the liposome.

Figure 3 shows graphs indicating the change in steady-state fluorescence intensity (panel A) and anisotropy (panel B) of safranine O as membrane potential is developed

across DOPC liposomes containing 0.39 mol % gramicidin A. Response follows influx of potassium ions after addition of liposomes to a solution of KI.

Figure 4 contains graphs showing the potential-induced decrease in fluorescence intensity as a result of the influx of potassium ions into unilamellar DOPC liposomes containing various levels of gramicidin A (a) in solution and (b) following entrapment in DGS derived silicate. Units are normalized as the ratio of intensity observed at time zero.

Figure 5 contains graphs showing the effect of different potassium iodide concentrations on the potential induced fluorescence response of safranine O for liposomes containing 0.39 mol % gramicidin A (a) in solution and (b) after entrapment in DGS derived silica. Inset for (a) depicts typical time trials for the various potassium iodide concentrations. Data are normalized as the ratios of final and initial fluorescence intensities.

Figure 6 is a graph showing the inhibition of potassium ion flux through DOPC liposomes containing 0.39 mol % gramicidin A as a result of adding various concentrations of calcium ions to liposomes in the presence of 3 M KI.

Figure 7 is a graph showing the aging of samples containing DOPC liposomes with 0.39 mol % gramicidin A after entrapment in DGS derived silica in the presence of 25 % glycerol in distilled deionized water (●), in distilled deionized water (■) or without any external buffer or solution (π). Data are normalized as a percentage of response observed on day 1.

Figure 8 shows the response obtained upon addition of ^{3}H-epibetadine to *Torpedo californica* nAChR entrapped in sodium silicate derived silica (Panel A) and the response obtained for blank liposomes entrapped in sodium silicate derived materials (Panel B).

Figure 9 shows the specific binding of ^{3}H-epibetadine to human nAChR when entrapped in DGS derived materials relative to the binding obtained in the absence of entrapped nAChR.

Figure 10 shows the results of a competitive binding assay wherein varying concentrations of a non-radioactive antagonist (d-tubocurarine, Panel A) or agonist (nicotine, Panel B) is introduced along with 2.5 nM ^{3}H-epibetadine to IMR-32

nAChR entrapped in DGS derived materials. The IC_{50} and K_I values are in good agreement with those obtained from solution based experiments.

Figure 11 shows the concept of the fluo-3 based assay for measuring the Ca(II) ion flux across nAChR doped liposomes. In the absence of an agonist the channel remains closed and no ion flux is observed. Upon binding of an agonist the nAChR ion channel opens and Ca(II) can pass into the membrane, resulting in a large increase in emission intensity from intraliposomal Fluo-3.

Figure 12 shows the changes in emission intensity of intraliposomal fluo-3 with time (Panel A) and the normalized concentration-dependent decrease in fluo-3 emission intensity (Panel B) due to blockage of the passage of Ca(II) ions upon addition of the antagonist d-tubocurarine to n-AChR doped liposomes entrapped in DGS derived glasses that were previously incubated with an excess of the agonist nicotine. The decease in emission intensity correlates to a decrease in ion flux owing to closing of the nAChR channel upon binding the antagonist.

Figure 13 shows the changes in emission intensity of intraliposomal fluo-3 with time (Panel A) and the normalized concentration-dependent decrease in fluo-3 emission intensity (Panel B) due to enhanced passage of Ca(II) ions upon addition of the agonist cytisine to n-AChR doped liposomes entrapped in DGS derived glasses that were previously incubated with an excess of the antagonist d-tubocurarine. The increase in emission intensity correlates to an increase in ion flux owing to opening of the nAChR channel upon binding the agonist.

Figure 14 is a graph showing the fluorescence intensity response of the calcium selective indicator dye Fluo-3 to the influx of calcium into DOPC liposomes in buffered solution following the addition of a calcium selective ionophore ionomycin to the membrane.

Figure 15 is a graph showing the response of fluo-3 to the addition of calcium ions for DOPC liposomes both with and without ionomycin present within the membrane following entrapment in sodium silicate derived silica.

Figure 16 are pictures showing a microarray of sol-gel entrapped liposomes containing ionomycin, and shows that our entrapment and signalling methods are amenable to the microarray format. Panel A shows the array before addition of calcium, panel B show the array after addition of calcium. Column 1 and 5 contain

Fluo-3 loaded DOPC liposomes with ionomycin; column 2 contains only buffered sodium silicate derived silica; column 3 contains entrapped fluorescein-dextran; column 4 contains fluo-3 loaded DOPC liposomes without ionomycin.

DETAILED DESCRIPTION OF THE INVENTION

(i) Method of Entrapping Membrane-Bound Proteins

Phospholipid liposomes with reconstituted ionomycin, gramicidin A or nAChR ion channels were readily incorporated into sol-gel derived silicates without loss of ion channel activity when the sol gel was prepared from protein- and membrane-compatible, silica precursors such as organic polyol-derived silanes and sodium silicate. Steady-state fluorescence measurements of the tryptophan residues of gramicidin A indicated that it remained in its native conformation within the phospholipid membrane following entrapment. It was also found that ion channel activity was retained for reconstituted gramicidin A and that this activity was still sensitive to various electrochemical gradients caused by potassium ion concentration. It was also established that ion flux could be inhibited by the presence of divalent cations; moreover, the ion flux activity through gramicidin channels was retained for several weeks. In addition, it was found that ion channel activity could be produced using either an ionophore (ionomycin) to produce Ca(II) flux across the membrane, or by using the ligand-gated ion channel (LGIC) nAChR, which produced agonist or antagonist dependent transmembrane fluxes of Ca(II).

Accordingly, the present invention relates to a method of immobilizing membrane-associated molecules in silica matrixes comprising combining a liposome assembly comprising the membrane-associated molecule with a protein- and membrane-compatible sol-gel precursor under conditions which allow a gel to form.

Membrane-associated molecules which may be immobilized using the method of the invention include, for example, non-natural ionophores, ion channel proteins, ion-channel receptors, G-protein coupled receptors or membrane associated enzymes. Some specific examples of proteins include gramicidin, bacteriorhodopsin and the nicotinic acetylcholine receptor. Other examples include: the cys-loop receptor subfamily of LGIC such as $GABA_A$, Glycine, GLUC1, $5\text{-}HT_3$ and nicotinic acetylcholine receptors; the ATP gated channel superfamily of LGIC receptors as well as the glutamate cationic receptor superfamily of LGIC receptors; G-protein coupled

receptors such as the dopamine receptor, histamine receptor and androgenic receptors; membrane transport proteins and membrane associated enzymes such as γ-glutamyltranspeptidase or lipase. Examples of non-natural ionophores, include, for example, various ionophore antibiotics such as ionomycin, monensin, lonomycin, laidlomycin, nigericin, grisorixin, dianemycin, lenoremycin, salinomycin, narasin, antibiotic X206, alborixin, septamycin, antibiotic A204, maduramicin and semduramicin, compound 47224, lasalocid (also including factors A, B, C, D and E), mutalomycin, isolasalocid A, lysocellin, tetronasin, echeromycin, antibiotic X-14766A, antibiotic A23187, antibiotic A32887, compound 51532 and K41 ionomycin, and any other non-natural molecules that act as membrane ion transporters. These lists are not exhaustive, but are meant to provide selected examples of the types of proteins and other membrane-associated molecules that may be used in the current invention. One of ordinary skill in the art would appreciate that other membrane-bound or membrane-associated molecules will also be amenable to the immobilization method described herein.

As used herein, the term "immobilized" means that the liposome assembly is physically, electrostatically or otherwise confined within the nanometer-scale pores of the biomolecule-compatible matrix. In an embodiment of the invention, the assembly does not associate with the matrix, and thus is free to rotate within the solvent-filled pores. In a further embodiment of the invention, the assembly is optionally further immobilized through electrostatic, hydrogen-bonding, bioaffinity, covalent interactions or combinations thereof, between the lipid bilayer and the matrix. In a specific embodiment, the immobilization is by physical immobilization within nanoscale pores.

The term "liposome assembly comprising the membrane-associated molecule" as used herein means that membrane-associated molecule is either extrinsically or intrinsically associated with the lipid components in the liposome though hydrophobic, electrostatic, hydrogen-bonding, bioaffinity, covalent interactions or combinations thereof. The membrane-associated molecule may be associated with the headgroups or acyl chains of the liposome or with both.

The term "a" as used herein, unless otherwise indicated, also denotes "one or more".

By "biomolecule-compatible" and "membrane compatible" it is meant that the silica matrix either stabilizes proteins, membranes and/or other biomolecules against denaturation or does not facilitate denaturation. The term "biomolecule" as used herein means any of a wide variety of proteins, enzymes, organic and inorganic chemicals, other sensitive biopolymers including DNA and RNA, and complex systems including whole or fragments of plant, animal and microbial cells that may be entrapped in the matrix.

In the invention, the biomolecule-compatible and membrane-compatible matrix is a sol-gel. In particular, the sol-gel is prepared using biomolecule- and membrane-compatible techniques, i.e. the preparation involves biomolecule- and membrane-compatible precursors and reaction conditions that are biomolecule- and membrane-compatible. In a further embodiment of the invention, the biomolecule-compatible sol gel is prepared from a sodium silicate precursor solution. In still further embodiments, the sol gel is prepared from organic polyol silane precursors. Examples of the preparation of biomolecule-compatible sol gels from organic polyol silane precursors are described in inventor Brennan's co-pending patent applications entitled "Polyol-Modified Silanes as Precursors for Silica", PCT patent application S.N. PCT/CA03/00790, filed on June 2, 2003 and corresponding U.S. patent application filed on June 2, 2003; and "Methods and Compounds for Controlling the Morphology and Shrinkage of Silica Derived from Polyol-Modified Silanes", PCT patent application S.N. PCT/CA03/01257, filed August 25, 2003 and corresponding U.S. patent application filed on August 25, 2003, the contents of all of which are incorporated herein by reference. In specific embodiments of the invention, the organic polyol silane precursor is prepared by reacting an alkoxysilane, for example tetraethoxysilane (TEOS) or tetramethoxysilane (TMOS), with an organic polyol. In an embodiment, the organic polyol is selected from sugar alcohols, sugar acids, saccharides, oligosaccharides and polysaccharides. Simple saccharides are also known as carbohydrates or sugars. Carbohydrates may be defined as polyhydroxy aldehydes or ketones or substances that hydroylze to yield such compounds. The organic polyol may be a monosaccharide, the simplest of the sugars, or a carbohydrate. The monosaccharide may be any aldo- or keto-triose, pentose, hexose or heptose, in either the open-chained or cyclic form. Examples of monosaccharides

that may be used in the present invention include one or more of allose, altrose, glucose, mannose, gulose, idose, galactose, talose, ribose, arabinose, xylose, lyxose, threose, erythrose, glyceraldehydes, sorbose, fructose, dextrose, levulose and sorbitol. The organic polyol may also be a disaccharide, for example, one or more of, sucrose, maltose, cellobiose and lactose. Polyols also include polysaccharides, for example one or more of dextran, (500-50,000 MW), amylose and pectin. In embodiments of the invention the organic polyol is selected from one or more of glycerol, sorbitol, maltose, trehelose, glucose, sucrose, amylose, pectin, lactose, fructose, dextrose and dextran and the like. In embodiments of the present invention, the organic polyol is selected from glycerol, sorbitol, maltose and dextran. Some representative examples of the resulting polyol silane precursors suitable for use in the methods of the invention include one or more of diglycerylsilane (DGS), monosorbitylsilane (MSS), monomaltosylsilane (MMS), dimaltosylsilane (DMS) and a dextran-based silane (DS). In embodiments, the polyol silane precursor is selected from one or more of DGS and MSS.

In further embodiments of the invention, the biomolecule-compatible matrix precursor is selected from one or more of functionalized or non-functionalized alkoxysilanes, polyolsilanes or sugarsilanes; functionalized or non-functionalized bis-silanes of the structure $(RO)_3Si\text{-}R'\text{-}Si(OR)_3$, where R may be ethoxy, methoxy or other alkoxy, polyol or sugar groups and R' is a functional group containing at least one carbon (examples may include hydrocarbons, polyethers, amino acids or any other non-hydrolyzable group that can form a covalent bond to silicon); functionalized or non-functionalized chlorosilanes; and sugar, polymer, polyol or amino acid substituted silicates.

In yet another embodiment of the present invention, the biomolecule compatible matrix further comprises an effective amount of one or more additives. In embodiments of the invention the additives are present in an amount to enhance the mechanical, chemical and/or thermal stability of the matrix and/or assembly components. In an embodiment, the mechanical, chemical and/or thermal stability is imparted by a combination of precursors and/or additives, and by choice of aging and drying methods. Such techniques are known to those skilled in the art. In further embodiments of the invention, the additives are selected from one or more of

humectants and other protein stabilizing agents (for e.g. osmolytes). Such additives include, for example, one or more of organic polyols, hydrophilic, hydrophobic, neutral or charged organic polymers, block or random co-polymers, polyelectrolytes, sugars (natural or synthetic), and amino acids (natural and synthetic). In embodiments of the invention, the one or more additives are selected from one or more of glycerol, sorbitol, sarcosine and polyethylene glycol (PEG). In further embodiments, the additive is glycerol.

In a particular embodiment of the invention biocompatible matrix is a silica based glass prepared from, for example, a silicon alkoxide, alkylated metal alkoxide or otherwise functionalized metal alkoxide or a corresponding metal chloride, silazane, polyglycerylsilicate, diglycerylsilane or other silicate precursor, optionally in combination with additives selected from one or more of any available organic polymer, polyelectrolyte, sugar (natural or synthetic) or amino acids (natural and non natural).The preparation of sodium silicate solutions for use as a sol-gel precursor is known in the art.[38] The use of sodium silicate as a sol-gel precursor may be problematic if either sodium or potassium ions are to be transported through the membrane of the liposome due to interference from the sodium ions present in the precursor solution. In these circumstances, it is preferred to use the organic polyol silane precursors described above. In the case of ligand-gated ion channels, the sodium may not enter the internal compartment of the liposome in the absence of ligand, accordingly the residual sodium could be washed away before use allowing sodium silicate to be a suitable precursor for the transport of sodium or potassium ions through these types of membrane associated molecules.

The liposome-molecule assembly can be prepared using methods known to those skilled in the art. Typically a solution of the membrane-associated molecule, either with or without its intrinsic lipids (if any) present, is combined with a solution of a suitable lipid. Any lipid which forms liposomes may be used, for example, phospholipids, such as 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC). Suitable lipid components may include, but are not limited to: phospholipids, sphingolipids, glycolipids, synthetic and non-natural lipids, fluorescently labelled lipids, polymer-linked and polymerizable lipids (i.e., diacetylenic lipids), photoreactive lipids, fatty acids, fatty amines and hydrophobic moieties such as cholesterols, sterols etc. These

may be used alone or in combination, and the resulting liposomes may contain mixtures of single or double chain surfactants, with chain lengths in the range of 4-30 carbons, with between 0 and 10 sites of unsaturation per chain. Upon formation of the lipid mixture, all organic solvents are removed (if necessary) and the resulting lipid films may be rehydrated in suitable buffer solutions followed by conversion to lipid vesicles (by sonication and/or extrusion, or any other suitable method) with the membrane associated molecule embedded within the lipid bilayer.

The liposome-molecule assembly may be combined with a protein- or membrane-compatible, sol-gel precursor solution under conditions which allow a gel to form. By "gel" it is meant a solution or "sol" that has lost flow. The sols lose flow due to the hydrolysis and polycondensation of the precursor. The hydrolysis and condensation of the polyol silane and sodium silicate precursors may suitably be carried out in aqueous solution. Suitably, a solution, for example a homogeneous solution, of precursor, in acidified water is used, or in the case of DGS a solution of the precursor in water or buffer at neutral pH. Sonication may be used in order to obtain a homogeneous solution. By "homogeneous" it is meant having an essentially uniform composition or structure. Conditions which allow the formation of a gel comprise adjusting the pH of the aqueous solution of precursor so that formation of a gel occurs. Suitably, the pH may be in the range of about 4 – 11. The pH may be adjusted, for example, by the addition of suitable buffer solutions or resins. As the solutions lose flow, they can be formed, cast, moulded, shaped, spun, pin-printed as microarrays or drawn into desired shapes. Examples of such shapes include, but are not limited to, films, fibres, monoliths, pellets, granules, tablets, rods or bulk. The solutions may also be placed into multi-well plates for high-throughput screening applications, or printed as microarrays for multianalyte sensing or screening. Accordingly, in an embodiment of the present invention, the method of immobilizing membrane-associated molecules in silica matrixes comprises:

(i) combining an aqueous solution of the protein- and membrane-compatible, sol-gel precursor with an aqueous solution of a liposome assembly comprising the membrane-associated molecule;

(ii) adjusting the pH of the combination of (i) so that it is in the range of about 4-11;

(iii) shaping the combination into a desired shape;

(iv) allowing the combination to gel; and

(v) aging and partially drying the gel.

A person skilled in the art would appreciate that the conditions may need to be adjusted depending on the identity of the sol-gel precursor and the liposome assembly and could do so without undue experimentation in light of the present disclosure and the examples provided herein.

Once the gel has been formed and shaped it may be aged over a period of time under select conditions to lock the conformation of the gel, its pores, matrixes and interconnecting channels into fixed positions and permit long term storage. In embodiments of the invention, the gels are aged in buffer or in a solution comprising an effective amount of a humectant, for example glycerol (suitably about 5-50% (v/v) of glycerol in water or buffer solution, preferably 25% (v/v) of glycerol in water or buffer solution).

In embodiments of the invention, the protein- and membrane-compatible, sol-gel precursor solution and the liposome assembly are combined in the presence of an indicator molecule. Alternatively, the liposome assembly further comprises an indicator molecule located on the interior of the liposome. As used herein, the term "indicator" molecule refers to any compound that may be used to detect a change in the membrane-associated molecule's conformation or activity, including trans-membrane ion fluxes. Examples of such indicator molecules include compounds which have at least one detectable characteristic which is sensitive to changes in, for example, pH, membrane potential, ionic strength, divalent ion concentration or the hydrophilicity/hydrophobicity of its environment. Specific examples of such an indicator molecules are the lipophilic cationic dye safranine O, the fluorescence of which is sensitive to changes in membrane potential, and the fluorescent dye fluo-3, which is sensitive to the concentration of free Ca(II) in solution.

In embodiments of the invention, the protein- and membrane-compatible, sol-gel precursor solution and the liposome assembly are combined in the presence of one or more ligands (natural or unnatural) for the protein (for example a receptor) in question, that may optionally be labelled, for example, fluorescently labelled, for detection of activity of the protein. The term "label" refers to any detectable moiety.

A label may be used to distinguish a particular ligand from others that are unlabelled, or labelled differently, or the label may be used to enhance detection.

Herein, the entrapment of the reconstituted ion channel peptide gramicidin A (gA) into sol-gel derived silica is reported along with the measurement of ion flux through the membrane using a novel fluorescence method based on the potential-sensitive probe Safranine O. Gramicidin A was chosen as a model system since the fluorescence properties of the tryptophan residues of gA can be measured to determine protein conformation and local environment in solution and upon reconstitution and entrapment.[49,50,51,52] The results clearly demonstrate that upon entrapment gramicidin remains embedded in the phospholipid membrane and that its ion channel activity is retained upon entrapment.

The entrapment of a reconstituted ionophore (ionomycin) and a ligand-gated transmembrane receptor (AChR) into sol-gel derived silica is also reported along with the measurement of Ca(II) ion flux through the membrane using a novel fluorescence method based on the Ca(II)-sensitive probe fluo-3. Ionomycin was chosen as a model system since the ionophore can be used to produce pores in the membrane to optimize fluorescence signals resulting from Ca(II) transmembrane ion flux. AChR was chosen as it is a pharmacologically relevant ligand-gated receptor that has the potential to be used as a drug target. The results clearly demonstrate that upon entrapment the AChR remains embedded in the phospholipid membrane and that its ion channel activity is retained upon entrapment.

The present invention further relates to protein- and membrane-compatible sol-gels with a liposome/membrane-associated molecule assembly immobilized therein and prepared using the method as described hereinabove.

(ii) Uses

The immobilization of membrane-associated molecules is important in several technologies including the development of biosensors, protein microarrays and bioaffinity columns. The sol-gels prepared using the method described in the previous section can be used for any of these applications. In particular, the gels may be used to screen for agonists, antagonists and modulators of any membrane associated molecule, such as non-natural ionophores, ion-channel receptors, G-protein coupled receptors or membrane-associated enzymes; microarraying of protein-

membrane complexes for high-throughput screening of modulators of membrane-bound receptors; or immobilization of membrane-bound receptors into sol-gel derived monolithic columns for drug screening by frontal-affinity chromatography with mass spectrometric detection.

Accordingly, also included within the scope of the present invention are methods for the detection of modulators of a membrane-associated molecule comprising:

(a) exposing a liposome assembly comprising the membrane-associated molecule, said assembly being immobilized in a protein- and membrane-compatible sol-gel, to one or more test substances; and

(b) detecting a change in one or more characteristics of the membrane associated molecule.

In embodiments of the invention, the protein- and membrane-compatible sol-gel is prepared using a method described herein. In further embodiments of the invention, a change in the one or more characteristics of the membrane-associated molecule in the presence of the one or more test substances compared to a control indicates that the one or more test substances are modulators of the membrane-associated molecule.

By "control" is meant repeating the same method, under the same conditions but in the absence of the one or more test substances.

The one or more test substances can be any compound which one wishes to test including, but not limited to, proteins (including antibodies), peptides, nucleic acids (including RNA, DNA, antisense oligonucleotide, peptide nucleic acids, RNA or DNA aptamers, ribozymes or deoxyribozymes), fragments of proteins, peptides, and nucleic acids carbohydrates, organic compounds, inorganic compounds, natural products, library extracts, bodily fluids and other samples that one wishes to test for modulators of the membrane-bound protein. The one or more test substance may be in liquid or gaseous form. Typically a solution of known concentration of the one or more test substances is employed.

In embodiments of the invention, the method for the detection of modulators of a membrane-associated molecule further involves a liposome assembly comprising a membrane-associated molecule in combination with other entities that facilitate the detection of modulation of the membrane-associated molecule by the one or more test

substances. In an embodiment of the invention the other entities are selected from one or more of indicator molecules and ligands (natural or unnatural) for the receptor protein being investigated. In embodiments of the invention, the ligands may be labelled or unlabelled.

The method of detecting modulators of membrane-associated molecules may be "miniaturized" in an assay system through any acceptable method of miniaturization, including but not limited to multi-well plates, such as 24, 48, 96 or 384-wells per plate, microfluidic chips, microarrays or slides. The assay may be reduced in size to be conducted on a microfluidic-chip support, advantageously involving smaller amounts of reagents and other materials. Any miniaturization of the process which is conducive to high-throughput screening is within the scope of the invention. The "one or more characteristics of the membrane-associated molecule" that may be used to detect modulators of the membrane-associated molecule include, but are not limited to, molecule-mediated transmembrane ion fluxes and conformational/environmental changes in the protein, membrane or a probe molecule that is associated with the protein or membrane, or entrapped within the liposome, or by binding of fluorescent or radioactive ligands by the entrapped protein.

In an embodiment of the invention the membrane-associated molecule is an ion channel protein or ionophore and the characteristic of the membrane-associated protein or ionophore that is detected is the flux of ions through the protein or ionophore from the exterior of the liposome to the interior. Such a flux or movement of ions results in the formation of an electrochemical potential across the liposome membrane and/or in the presence of a specific ion within the liposome. Certain fluorescent indicator molecules, for example the lipophilic cationic dye safranine O, respond to the development of membrane potential by partitioning to certain locations in the assembly resulting in either an increase or decrease in fluorescence intensity and anisotropy. Other dyes, such as fluo-3, respond to the presence of specific ions, such as Ca(II), resulting in a large increase in fluorescence intensity. Modulation of this change in fluorescence intensity and/or anisotropy by the one or more test substances can be used as a means to detect modulators of membrane-associated molecules.

In an embodiment of the invention, the sol-gel entrapped liposomes comprising membrane associated molecules are formed into microarrays. Microarrays may be formed by pin-printing the solution comprising the liposome assembly and the sol-gel precursors onto a suitable surface in array format before the solution gels. The solutions are then allowed to gel and dry on the surface. Suitable methods for forming sol-gel microarrays are known in the art (see, for example, inventor Brennan's co-pending PCT and U.S. regular applications entitled "Multicomponent Protein Microarrays", filed on November 3, 2003). The present invention provides the first example of the use of transmembrane ion flux as a signalling method for microarrays.

Fluorescence is only one of many means of detecting change in one or more characteristics of the membrane-associated molecule. Because of the light-transmission capabilities of the matrixes of the present invention, UV, IR and visible light optical spectroscopy, as well as luminescence, adsorption, emission, excitation and reflection techniques are all suitable for detecting changes in the characteristics of the entrapped membrane associated molecule.

The present invention also includes kits, biosensors, microarrays, chromatographic and bioaffinity columns comprising the silica matrixes comprising a liposome-protein assembly prepared as described herein.The kits of the present application comprise, in different combinations, the matrixes, reagents for use with the matrixes, signal detection and processing instruments, databases and analysis and database management software above. The kits may be used, for example, to determine the effect of one or more test compounds on a membrane-associated molecule and to screen known and newly designed drugs.

Yet another aspect of the present invention provides a method of conducting a target discovery business comprising:

(b) providing one or more assay systems for identifying test substances by their ability to effect one or more membrane-associated molecules based systems, said assay systems using a method of the invention;

(b) (optionally) conducting therapeutic profiling of the test substances identified in step (a) for efficacy and toxicity in animals; and

(c) licensing, to a third party, the rights for further drug development and/or sales or test substances identified in step (a), or analogs thereof.

(iii) Improved Method for Detecting Membrane Potentials

A novel procedure amenable to the sol-gel method of entrapment was developed to monitor ion flux through an entrapped membrane-associated molecule. In this method, the fluorescence indicator used to detect the development of a potential across the lipid membrane or the presence of a specific ion inside the liposome due to ion flux was located on the inside of the liposome assembly only. Literature methods describe the use of fluorescent indicators in the external solution. Problems caused by interactions of the indicator molecule with the anionic surface of the silica can arise when the indicator molecules are in the external solution. Such problems are avoided when the indicator molecules are located within the interior of the liposome assembly since in this location these molecules are not able to interact with the silica surfaces. Accordingly, the present invention further relates to a method for preparing a sol gel immobilized liposome assembly comprising a membrane associated molecule, wherein the membrane-associated molecule is an ion-channel molecule, comprising:

(a) obtaining a solution of the liposome assembly having an indicator molecule located on the interior of the assembly;

(b) removing the indicator molecule from solution external to the liposome assembly; and

(c) combining the liposome assembly solution with a silica precursor solution under conditions which allow a gel to form.

The present invention also relates to an improved method for the detection of membrane potentials in a sol-gel immobilized liposome assembly comprising a membrane-associated molecule, wherein the membrane-associated molecule is an ion-channel molecule, comprising:

(a) obtaining a solution of the liposome assembly having an indicator molecule located on the interior of the assembly;

(b) removing the indicator molecule from solution external to the liposome assembly;

(c) combining the liposome assembly solution with a silica precursor solution under conditions which allow a gel to form;

(d) contacting the gel with the ion and optionally a test substance; and

(e) detecting a change in the indicator molecule upon transmembrane ion flux.

In embodiments of the invention the indicator molecule can be any compound that interacts with the surface of the sol gel, for example, the lipophilic cationic dye, safranine O. In further embodiments, the indicator molecule acts by detecting the ion directly upon entry into the interior of an entrapped liposome, for example the calcium dependent fluorophore, fluo-3. In still further embodiments, the indicator molecule is removed from the solution external to the protein-liposome assembly using dialysis or gel filtration chromatography.

In embodiments of the invention, the silica precursor is biomolecule- and membrane-compatible. In still further embodiments, the liposome assembly further comprises a ligand (natural or unnatural, labelled or unlabelled) for the membrane associated molecule (for example a receptor).

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

Materials

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), Egg phosphatidylcholine (EggPC), Egg Phosphatidylethanolamine (EggPE) and sphingomylin were purchased from Avanti Polar-Lipids, Inc (Alabaster, AL). Gramicidin A, High Purity (95%) was purchased from Calbiochem (San Diego, CA). The human nicotinic acetylcholine receptor was purchased from Perkin Elmer Life Sciences (Boston, MA) while *Torpedo californica* nAChR was purified from the electric organ of the organism according to established protocols.[53] Diglyceryl silane (DGS) was provided by Dr. Michael Brook of McMaster University and was prepared by a method that is described elsewhere.[54] The fluorescent dye Fluo-3 was purchased from Molecular Probes (Eugene, OR). The fluorescent dye safranine O, sodium silicate solution, Sephadex G25, sucrose, ethylenendiaminetetraacetic acid (EDTA), SM-2 Biobeads, Pottasium phosphate, ethylenediaminetetraacetic acid (EDTA), ethyleneglycolamine-tetraacetic acid (EGTA), potassium chloride, sodium aside, ionomycin, iodoacetamide, (-)-nicotine, cytisine, d-tubocurarine, phenylmethanesulfonylfluoride

(PMSF), N-dodecyl-b-D-manopyranoside (DMM), Asolectin® and polymethacrylate fluorimeter cuvettes (transmittance curve C) were obtained from Sigma (St. Louis, MO). Black and transparent ninety-six well microwell plates were purchased from Nalge Nunc International (Rochester, NY). ^{3}H-epibatidine was purchased from Amersham Biosciences (Buckinghamshire, UK). *Torpedo californica* electroplax was purchased from Aquatic Research Consultants (San Pedro, Cal.). Dialysis Tubing with a molecular weight cut-off of 3500 Da was purchased from Spectrum Laboratories Inc. (Rancho Domingez, CA). All water was twice distilled and deionized to a specific resistance of at least 18 MΩ.cm using a Milli-Q Synthesis A10 water purification system. All other chemicals were of analytical grade and were used without further purification.

Methods

Preparation of Fluo-3 Loaded Liposomes

DOPC stock solutions were purchased in chloroform at a concentration of 20 mg.mL^{-1}. DOPC stock solution was dispensed in disposable glass vials and the organic solvent was removed by evaporation under a dry nitrogen gas stream to remove the bulk of the organic solvent, followed by evaporation under vacuum for two hours. The resulting dried lipid films were then rehydrated in a buffer consisting of 25 mM EGTA, 25 mM EDTA, 10% Sucrose (w/w), 10 mM KCl, 0.529 mM Fluo-3, pH 7.6 to a final lipid concentration of 2mg/mL. The hydrated liposomes were then extruded through 600 nm pores, with an Avanti – MINIEXTRUDER at room temperature to create a mono-disperse suspension of unilamellar liposomes 600 nm in diameter. The external Fluo-3 was removed by filtration through a column packed with Sephadex G25 to yield liposomes with dye only on the interior of the liposome.

Preparation of Reconstituted Gramicidin:

The Gramicidin A stock was prepared in a solution of chloroform, trifluoroethanol and dimethylsulfoxide (19:5:1 volume ratio) to a final concentration of 4.79 x 10^{-4} M. Gramicidin A stock solutions were mixed with the lipid stock solutions (20 mg.mL^{-1} DOPC in chloroform) in disposable glass vials to provide final ratios of gA:lipid of either 0.39 mol% or 0.94 mol%. The organic solvent was removed by evaporation under a dry nitrogen gas stream to remove the bulk of the organic solvent, followed by evaporation under vacuum for two hours. The resulting

dried gA:lipid films were then rehydrated in an appropriate buffered solution followed by high frequency sonication for one hour with a VWR Scientific Aquasonic Model 50T sonicator to create small unilamellar phospholipid vesicles with reconstituted Gramicidin A ion channels.[55,56,57]

For ion-channel studies, reconstitution of gramicidin A samples was done using an unbuffered aqueous solution containing 50 μM safranine O at pH 7.0. To remove the safranine O from the exterior of the liposomes, samples were dialyzed in distilled deionized water until negligible safranine O fluorescence was observed in the dialysate. Liposome solutions that were used for tryptophan fluorescence studies consisted of 0.94 mol% of gramicidin A in DOPC that was hydrated to final concentrations of 1.0 μM of gA in 100 mM phosphate buffer at pH 7.0.

Preparation of Liposomes Containing Nicotinic Acetylcholine Receptor (nAChR)

Lipid films consisting of EggPC, EggPE, Sphingomylin and cholesterol in mol ratios of 55:27:9:9 percent, respectively, were prepared as described earlier, and rehydrated to a final lipid concentration of 3.0 mg/mL and Fluo-3 concentration of 65 μM. The liposome stock was then mixed with a small amount of DMM bringing the solution to a final concentration of 0.2 mM. Immediately following this, IMR-32 nAChR stock was added to the detergent lipid mixture and allowed to incubate at 4 C for 90 min. Following this 15 mg of SM2-Biobeads were added to the mixture and incubated for 1 hr. This was repeated 3 times with the final addition allowed to incubate for a period of 12 hrs. The nAChR containing liposomes were then passed through a Sephadex G25 column to remove any additional detergent as well as the extraliposomal Fluo-3. The IMR-32 nAChR containing liposomes were used for ion flux studies without further modification.

Entrapment of Reconstituted Gramicidin:

Diglyceryl silane (DGS) derived sol-gels were prepared by adding 0.212 g of solid DGS and 5 μL of 0.1 N HCl to 650 μL of distilled deionized water followed by sonication at 0 C for 1.5 hours until all of the silane precursor had been dissolved and the solution had become homogeneous and transparent. Samples used for the collection of Trp spectra were prepared by placing 70 μL of the solution in the well of a microwell plate. For ion channel studies, the dialysed liposomes were mixed in a 1:1 volume ratio with DGS, along with 2 μL of 1M NaOH and 4 μL of 2.5 M NaCl in

a microwell plate to a final volume of 76 μL. Sodium hydroxide and sodium chloride were added only to allow gelation to occur, and were not present at a sufficient concentration to produce a significant effect on the flux of potassium ions across the membrane. For Safranine O fluorescence anisotropy studies, thin silica films were prepared by mixing the hydrolyzed DGS precursor solution with the liposome solution in a 1:1 volume ratio to a final volume of 100 μL. The solution was then spin-cast onto a glass slide (8 x 32 mm) for 1 min at a rate of 2000 rpm. In all cases, the samples were allowed to gel and were then aged in air (dry-aged), in buffer (wet-aged) or in a 25% solution of glycerol in water (glycerol-aged) for 1 to 28 days before fluorescence measurements were done.

Entrapment of DOPC liposomes and reconstituted nAChR:

Sodium silicate or DGS derived sol precursors were prepared by methods described previously.[58] The sol solution was mixed 1:1 (v/v) with the buffered liposome or reconstituted nAChR solution, in the bottoms of 96 well microtiter plates to a final volume of 100 μL. For entrapment of IMR-32 cells for ion flux assays, the nAChR containing stock described above was uses for entrapment without further dilution. For radioassays using IMR-32 nAChR, the stock sample as provided directly from Perkin Elmer was diluted four fold in 25mM HEPES, 100mM KCl, 5 mM EGTA, pH 7.4 and mixed 1:1 (v/v) in DGS derived sol. The samples were aged for 1 hr at 4 C. For Torpedo Californica nAChR, the stock sample was diluted four fold in 150 mM HEPES, 100 mM KCl, 5 mM EDTA, pH 7.4, and mixed 1:1 (v/v) with sodium silicate derived sol.

Steady State Tryptophan-Fluorescence Measurements of gA:

Fluorescence measurements were performed using a Jobin Yvon-SPEX Fluorolog-3 Model 212 T-format spectrofluorimeter (ISA Instrument Int. Edison, NJ) with a MicroMAX 96-well fluorescence plate-reader attachment that was interfaced to the spectrofluorimeter using a bifurcated fused silica optical fiber. Tryptophan emission spectra of reconstituted Gramicidin A were collected from 310 to 450 nm using an excitation wavelength of 280 nm. All spectra were collected in 0.5 nm increments using 5 nm bandpasses on the excitation and emission monochromators and an integration time of 0.5 s per point. Appropriate blanks were subtracted from

each sample and the spectra were corrected for the wavelength dependence of the emission monochromator and photomultiplier tube.

Ion-channel Activity Assay of gA:

The fluorescence intensity response of safranine O was monitored using the MICROMAX microwell plate reader. Fluorescence emission was monitored as a function of time at 565 nm with an excitation wavelength of 528 nm upon addition of 125 μL of various concentrations of potassium iodide to the top of the monolithic samples in the microwell plate to create an ionic gradient across the membrane. Emission measurements were performed over a period of 125 s (solution experiments) or 600 s (for entrapped gA) using 0.25 sec intervals with a 0.20 sec integration time and emission and excitation bandpasses of 5nm. The responses were normalized to the intensity value obtained before addition of the salt solution. Alternatively, fluorescence anisotropy was monitored in the T-format in 1 second intervals with a 0.95 s integration time over a period of 125 s after addition of KI solutions. All anisotropy measurements were done using glass slides with spin-coated films of silica which were mounted at an angle of 55 with respect to the excitation beam (90 geometry). All anisotropy measurements were corrected for the instrumental G factor to account for any polarization bias in the monochromators.

Radioassays of nAChR:

Samples of either entrapped *Torpedo californica* nAChR, IMR-32 nAChR or Asolectin liposomes were formed in the bottoms of 96-well microtiter plates. 100 μL of nAChR stock (3.72 nmol/mg protein *Torpedo californica* nAChR or 168 fmol/mg IMR-32 nAChR) or 20 mg/mL Asolectin liposomes were mixed with an equal volume of DGS or sodium silicate sol in the well of a microwell plate, where formation of a solid gel commenced. The monoliths were allowed to cure for 1 hour, following which 10 μL of either buffer or 10 mM nicotine was added and allowed to incubate at 4 C for 2.5 hrs. 160 μL of ^{3}H-epibatidine in buffer was added to the monoliths to a final concentration of 1.0 – 3.0 nM, and incubated for 18 hr at 4 C. After incubation, 155 μL of ^{3}H-epibatidine solution was drawn off the top of the monolith and dissolved in 20 mL of Liquiscint scintillation fluid. The radioactive decay from ^{3}H-epibatidine was then counted for 5 min to determine the ratio of free ligand existing in solution. Nicotine was added to determine the amount of specific

binding to the receptor itself, and the Asolectin liposome samples were used to evaluate the amount of non-specific binding to the matrix. Using the information from these samples the amount of receptor bound ligand could be determined. For competitive assays seen in Figures 10 A and 10 B, 10 μL various concentrations of either (-)-nicotine or d-tubocurarine were added to the tops on the DGS monoliths containing IMR-32 nAChR or Asolectin liposomes and allowed to incubate for 2.5 hrs. 160 μL of 3.0 nM ^{3}H-epibatidine was then added and the samples were incubated for 18 hrs. Free ligand was determined as described above.

Ion-channel activity of IMR-32 nAChR

IMR-32 nAChR was used for the Fluo-3 based assays due to its increased calcium permeability as compared to the nAChR derived from *Torpedo californica.* In these assays, IMR-32 nAChR containing liposomes with an intraliposomal solution of Fluo-3 were entrapped as described above in 1:1 (v/v) in DGS derived silica. The buffered sol was then dispensed in the bottoms of standard 96-well microwell plates and allowed to cure for 1 hr at 4 C. Antagonism of the nAChR ion channel was measured by addition of 25 μL of the nAChR antagonist d-tubocurarine to the top of the nAChR-containing monolith in the 96-well plate. Ligand-gated ion-flux was monitored through time dependent changes in fluorescence intensity upon the addition of 50 μL of 3 M $CaCl_2$ using a TECAN-Safire microwell platereading fluorescence system. Fluo-3 emission was monitored and 526 nm with an excitation wavelength of 488 nm, emission and excitation bandpasses of 5 and 7.5 nm, and a detector gain of 130 V, over a period of 45 min. Similarly, agonism of the nAChR ion-channel was monitored using the same assay except the channel was first antagonized by incubating the samples in 0.012 M d-tubocurarine, which was then followed by the addition of various concentrations of (-)-cytisine along with 3 M $CaCl_2$. The time dependent responses were then normalized as a function of their initial fluorescence intensity before the addition of calcium as seen in Figure 12. The normalized changes in fluorescence intensity for the various concentrations of agonist or antagonist were then scaled as a percentage between their maximum and minimum response and an apparent dissociation constant could be determined by fitting the response to the "Hill" equation.

Microarray Experiments:

Sodium silicate derived sol precursors were prepared by methods described previously.[59] The sol solution was mixed 1:1 (v/v) with the buffered solution of ionomycin doped DOPC liposomes in the bottoms of 96 well microtiter plates to a final volume of 80 μL.

A Virtek Chipwriter Pro (Virtek Engineering Sciences Inc., Toronto, ON) robotic pinspotter equipped with a SMP 3 Stealth microspotting pin (Telechem Inc., Sunnyvale, CA) was used to print the ionmycin:liposome samples onto glass microscope slides from 96-well plates. Printing temperature was ambient with a humidity of approximately 50-70%. Completion of an array of 25 spots (5 x 5) took about 1 minute to perform, including pin wash cycles when using a printhead speed of 16 mm/s. Fluorescence images of the microarrays were taken with an Olympus BX50 Microscope equipped with a Roper Scientific Coolsnap Fx CCD camera using a multi-line argon ion laser source for excitation of fluo-3 (488 nm). Arrays containing the ionomycin doped DOPC liposomes were imaged before and 1 minute after addition of 1 mM Ca(II) using a 30 second integration time per image.

Example 1: Tryptophan Fluorescence of gA

The emission of Trp residues within proteins has been widely used to probe the conformation and dynamics of proteins within sol-gel derived silica.[60,61,62] In the case of gramicidin A, each homodimeric subunit of the ion channel contains four tryptophan residues, which NMR and crystallographic data have shown to be buried within the lipid bilayer.[63] Furthermore, the tryptophan residues of gramicidin have been shown to have distinctly different fluorescence emission spectra when located in the bilayer relative to being in solution.[49] The fluorescence emission properties of gA can therefore be used to indicate if gramicidin has survived the entrapment process and remained in the bilayer.

Figure 1 shows the emission spectra of gramicidin A before and after reconstitution into phospholipid vesicles comprised of DOPC, both in solution and after entrapment into DGS derived silicate. The results clearly show that the emission maximum of gramicidin embedded in DOPC liposomes stays constant at 340 nm in solution and in DGS derived silicate; whereas gramicidin in the absence of liposomes is red-shifted, with a peak emission intensity at 350 nm both in solution and when

entrapped. These results show that the gA remains within the bilayer structure when entrapped into DGS derived silicate.

It should be noted that attempts to entrap reconstituted gA into TEOS derived materials were unsuccessful, and generally led to fluorescence spectra that were consistent with aggregation of the gA peptide, and also produced a system that was not able to generate ion fluxes (results not shown). This is likely due to the loss of bilayer lipid membrane integrity resulting from the presence of ethanol,[33] which is a byproduct of the hydrolysis of TEOS. On the other hand, the use of the diglyceryl silane precursor, which liberates glycerol as a byproduct of hydrolysis, was able to retain the emission properties of reconstituted gA upon entrapment, and as discussed below, also provided an environment that was conducive to maintaining the ion-channel activity of entrapped gA.

Example 2: Ion Channel Activity of gA

The lipophilic cationic dye safranine O was used to follow the development of an electrochemical potential of K^+ across the phospholipid membrane. As shown in Figure 2, the changes in emission properties depend on whether the probe is located inside or outside of the membrane. As shown in Figure 2a, upon addition of KCl or KI to a membrane with the probe in the external solution, the influx of potassium ions through gA into the interior of the liposomes, combined with the exclusion of chloride ions, creates an electrochemical gradient across the membrane that is net positive on the interior and net negative on the exterior. Safranine O responds to development of such a membrane potential by partitioning into the hydrophobic lipid core due to the electrostatic attraction of the dye to the net-negative side of the membrane.[64,65,66] The net effect is to produce an increase in both fluorescence intensity and anisotropy as K^+ enters the membrane, owing to a reduction of collisional quenching of the dye and a decrease in the dynamic motions of the dye upon entry into the bilayer lipid membrane.[67,68]

Initial attempts to monitor ion channel activity using safranine O in the external solution were successful for reconstituted gA in solution. However, significant problems arose when the assay was attempted for reconstituted gA that was entrapped in DGS-derived glasses. For example, the dye was observed to have irreproducible responses from sample-to-sample, likely owing to direct interactions of

the cationic dye with the anionic surface of the silica which precluded association of the probe with the membrane.[69] Furthermore, the addition of KCl to the sol often led to leaching of some of the dye, further interfering with the response of the dye to membrane potential and leading to the need to include the probe within the KCl solution to avoid dilution of the probe.

To overcome these problems, it was necessary to locate the safranine O within the interior of the liposome only, as shown in Figure 2b. In this case, the influx of potassium ions again results in a positive interior and a negative exterior for the liposomes. However, the safranine O will now respond by partitioning from the membrane into solution owing to repulsion by the net-positive charge, leading to a decrease in both fluorescence intensity and anisotropy upon formation of an ion gradient. This assay format avoids association of the dye with the silica, and has the added advantage of allowing the liposomes to be formed with no internal salt so as to maximize the ion gradient that can be generated upon addition of a salt solution. A further alteration of the assay was to use potassium iodide in place of potassium chloride to generate the ion gradients. Iodide is a well-known quencher that is membrane impermeable, thus iodide abolishes any contribution to the fluorescence intensity from residual safranine O that is on the exterior of the liposome, enhancing the overall response from the probe that is located inside the membrane.

Figure 3 shows the changes in both fluorescence intensity (Panel A) and anisotropy (Panel B) that were obtained for reconstituted gA within DGS derived silicate upon addition of low and high levels of KI. Both the intensity and anisotropy decrease upon addition of KI, with the magnitude of the decrease becoming larger at the higher level of KI, as expected. These responses are consistent with the repulsion of the dye from the hydrophobic membrane owing to the influx of K^+ into the membrane, and provide evidence that ion channel activity can be monitored for reconstituted ion channels even after entrapment into sol-gel derived silica, proving that both the membrane and the ion channel are able to withstand the entrapment conditions.

To examine the effects of immobilization on the ion channel activity of gramicidin A, ion flux was monitored for reconstituted gA both in solution and after entrapment to allow a direct comparison of the fluorescence responses. For assays

performed in solution, liposomes that contained gA and an internal solution of safranine O were added to solutions of KI, and the changes in emission intensity were immediately measured. This method avoided dilution of the sample, as would occur if KI were added to a liposome solution, making it possible to accurately determine the initial intensity of the solution before the ion flux began. In the case of entrapped gA, the KI was added to the top of the monolith within the microwell plate to initiate a response. In this case, the liposomes were not diluted and thus the determination of the initial intensity was straightforward.

Figure 4 shows the response of safranine O to development of membrane potential for liposomes that contained varying levels of gA, both in solution and following entrapment. Even in the absence of gA, there is a significant fluorescence response that is due to the passive transport of K^+ directly through the lipid membrane. However, it is apparent that incorporation of gramicidin A into the phospholipid membrane results in development of a much larger potential at much faster rates over the time-course of the experiment, and that the response is increased in rate and magnitude as the level of gA increases. The results clearly show that entrapped gA exhibited a similar response to the development of membrane potential as those in solution, except that the rate and the final magnitude of the response were lower for the entrapped sample, likely owing to diffusional limitations for transport of K^+ into the membrane, and a lower overall level of free K^+ within the glass owing to electrostatic interactions with the anionic surface of the silica.[70]

After establishing that incorporation of reconstituted gramicidin A into the membrane resulted in a viable system for the generation of transmembrane ion fluxes, further investigations were carried out to examine the effects of different potassium ion concentrations on the development of the membrane potential. Figure 5 shows the response of DOPC liposomes containing 0.93 mol % gramicidin to a range of K^+ concentrations. As expected, the rate at which the emission intensity changes and the extent of the overall fluorescence response both increased as higher salt concentrations were introduced. Both solution and sol-gel entrapped samples exhibited the same trend of a concentration-dependent increase in response; however, the maximal response from the entrapped samples was again slightly lower than that measured in solution, in agreement with the results presented above.

Example 3: Inhibitors of gA Ion Channel Activity

A final test of the potential utility of the entrapped gA ion channel was to assess whether the ion channel activity could be inhibited by addition of channel blocking agents. It has been well established that the presence of divalent cations inhibits the flux of potassium and sodium ions through gramicidin by blocking their passage through the channel.[71] Inhibition of reconstituted gA entrapped in DGS derived silicate was examined by adding various levels of $CaCl_2$ to the entrapped samples along with 3.0 M KI. As shown in Figure 6, the presence of calcium ions produces a significant and concentration-dependent decrease in the potential induced fluorescence response to ion flux, consistent with inhibition of the ion-channel activity. The inhibitory effect requires the presence of several hundred millimolar of Ca^{2+}, which in expected given that Ca^{2+} must compete with molar levels of K^+ for access to the ion channel. A benefit of the "inverted" safranine O assay is that it avoids the potential for the direct interaction of Ca^{2+} with the fluorescent probe. Akerman et al have demonstrated the addition of divalent cations can directly inhibit the ability of safranine to embed into the membrane. However, entrapping the dye within the liposome leads to the exclusion of Ca^{2+} from the vicinity of the probe. Therefore, any change in response of safranine resulting from the presence of divalent cations cannot be a direct effect of the ions alone but must be due to inhibition of ion passage through the gramicidin ion channel. To confirm this assertion, the same assay was performed without incorporation of gramicidin into the membrane. The results confirmed that no decrease in response occurs upon addition of $CaCl_2$, ruling out direct interactions of Ca^{2+} with safranine O.

Example 4: Stability of Entrapped gA Ion Channel Protein

A key advantage of entrapping biomolecules is the potential for improving the long-term stability of the biomolecule.[33] To characterize the stability of entrapped gA, several samples were aged at 4 C over a period of several weeks in air, in the presence of external aqueous buffer, or in the presence of 25% glycerol. Figure 7 shows the effects of the various aging conditions on the response of safranine to the development of a transmembrane potential. The results show that dry-aged samples lost almost half of their initial activity after two days, and approximately 75% of the initial activity after three days of aging. The instability upon aging in air is an

obvious result of loss of water from the system, which leads to dehydration and rupture of the liposomes. On the other hand, samples that were aged either in buffer or in the presence of glycerol maintained their initial activity for over a week, suggesting that the entrapped ion channel may be sufficiently stable to allow the development of protein microarrays or bioaffinity columns that can be used for screening of agonists and antagonists of membrane-bound proteins.

Example 5: Radioassays of ligand binding by entrapped nAChR

To assess the ligand binding activity of entrapped nAChR we examined both IMR-32 and *Torpedo californica* nAChR when entrapped in either DGS (IMR-32 nAChR) or sodium silicate (Torpedo californica nAChR) derived silica. Figure 8 shows the response obtained upon addition of the radioligand ^{3}H-epibetadine to *Torpedo californica* nAChR entrapped in sodium silicate derived silica (Panel A) and the response obtained for blank liposomes entrapped in sodium silicate derived materials (Panel B). The results clearly show that there is statistically significant specific binding to the receptor, although the non-specific binding of the radioligand to the silica surface remains a problem. The total specific binding of the entrapped receptor (ca. 1000 cps) is approximately 25% the specific binding activity obtained for free AChR (ca. 4000 cps), indicating that a significant fraction of the entrapped nAChR is either denatured or inaccessible to analyte. However, the amount of specific binding is more than sufficient to conclusively prove that a fraction of the receptor remains active after entrapment.

Figure 9 shows the specific binding of ^{3}H-epibetadine to IMR-32 nAChR when entrapped in DGS derived materials relative to the binding obtained in the absence of entrapped nAChR. In this case the amount of specific binding is ca 500 cps, which is about half the amount observed for nAChR in sodium silicate glasses. No activity was observed from Torpedo californica nAChR in DGS derived glasses, suggesting that sodium silicate based materials may be superior for entrapment of nAChR.

Figure 10 shows the results of a competitive binding assay wherein varying concentrations of a non-radioactive antagonist (d-tubocurarine, Panel A) or agonist (nicotine, Panel B) were introduced along with a constant concentration of ^{3}H-epibetadine to IMR-32 nAChR entrapped in DGS derived materials. In each case the

residual radioactivity resulting from bound radioligand was decreased as the concentration of non-radioactive ligand increased, as expected. More importantly, the IC_{50} and K_I values for both d-tubocurarine and nicotine are in good agreement with those obtained from solution based experiments, and are in relatively good agreement with literature values, showing that the entrapment process does not dramatically alter the dissociation constants for the entrapped nAChR. The key drawback of the radioligand binding assay is that a similar response (i.e., decrease in radioactivity) is observed upon binding of either agonists or antagonists, and thus no discrimination of the functional response of the nAChR to such ligands can be done. To overcome this problem an assay based on enhancement and diminution of ion channelling was developed to provide more detailed information on the mode of action of the ligand, as described below.

Example 6: Modulation of nAChR ion channelling by an antagonist

Figure 11 shows the concept of the fluo-3 based assay for measuring the Ca(II) ion flux across nAChR doped liposomes, which is based on the enhancement in the emission intensity of fluo-3 upon binding of Ca(II). In the absence of an agonist the channel remains closed and no ion flux is observed. Upon binding of an agonist the nAChR ion channel opens and Ca(II) can pass into the membrane, resulting in a large increase in emission intensity from intraliposomal Fluo-3.

Figure 12 shows the changes in emission intensity of intraliposomal fluo-3 with time (Panel A) and the normalized concentration-dependent decrease in fluo-3 emission intensity (Panel B) due to blockage of the passage of Ca(II) ions upon addition of varying levels of the antagonist d-tubocurarine to n-AChR doped liposomes entrapped in DGS derived glasses that were previously incubated with an excess of the agonist nicotine to cause channel opening. The decease in emission intensity correlates to a decrease in ion flux owing to closing of the nAChR channel upon binding the antagonist. The results show that in the absence of antagonist, the presence of nicotine produced the expected rapid increase in fluorescence intensity upon addition of Ca(II). However, in the presence of the antagonist d-tubocurarine, the response is reduced owing to the blockage of a portion of the AChR ion channels. The signal eventually reaches the same intensity plateau as is observed for liposomes containing no nAChR, indicative of full blockage of the ion channel. As shown in

Panel B, the response is concentration-dependent, and thus will be useful for screening of antagonists against nAChR. This response indicates that the entrapped AChR channel can be modulated by antagonists, showing that the AChR:liposome assembly entrapped in sol-gel glass is suitable for drug-screening studies.

Example 7: Modulation of Entrapped AChR Ion Gating using an Agonist

Figure 13 shows the changes in emission intensity of intraliposomal fluo-3 with time (Panel A) and the normalized concentration-dependent decrease in fluo-3 emission intensity (Panel B) due to enhanced passage of Ca(II) ions upon addition of the agonist cytisine to nAChR doped liposomes entrapped in DGS derived glasses that were previously incubated with an excess of the antagonist d-tubocurarine. The increase in final emission intensity upon addition of Ca(II) in the presence of higher levels of cytisine correlates to an increase in ion flux owing to opening of the nAChR channel upon binding the agonist. Panel B shows that the increase in intensity occurs in a manner that depends on the concentration of cytisine added. The increase in ion flux provides clear evidence that the cytisine acts as an agonist and thus opens the AChR ion channel, producing a Ca(II) flux across the membrane. On the other hand, the decrease in signal upon addition of d-tubocurarine, described in Example 6, provides evidence that this ligand acts as an antagonist. These results prove that the ion-flux assay can discriminate agonists from antagonists and also proves that nAChR remains active upon entrapment and capable of generating transmembrane ion fluxes in an agonist- or antagonist-dependent manner.

Example 8: Ca(II) Ion Flux Measurements using Ionomycin:Liposome Assemblies

Figure 14 shows the fluorescence intensity response of the calcium selective indicator dye Fluo-3 to the influx of calcium into DOPC liposomes in buffered solution following the addition of a calcium selective ionophore ionomycin to the membrane. In this case, Ca(II) is initially present only outside the liposome, while fluo-3 is present only inside the liposome. The addition of ionomycin results in the incorporation of the ionophore into the membrane, and produces a channel through which Ca(II) can move into the interior of the liposome. The movement of Ca(II) into the liposome causes the fluo-3 response to increase dramatically (3-fold), producing some hyperpolarizability of the membrane, followed by a slight reduction in intensity

as the Ca(II) concentration equilibrates across the membrane. This example clearly shows that the entrapped dye can be used to monitor Ca(II) ion flux.

Figure 15 shows the response of fluo-3 to the addition of calcium ions for DOPC liposomes both with and without ionomycin present within the membrane following entrapment in sodium silicate derived silica. The data clearly show that the presence of ionomycin results in the formation of a pore within the lipid membrane, which in turn produced a flux of Ca(II) from the exterior to the interior of the liposome upon addition of Ca(II) to the entrapped liposome. The data confirm that the ionomycin-liposome-Ca(II) system can be used either for detection of ionomycin or detection of Ca(II) using transmembrane Ca(II) flux, and the resulting fluorescence intensity change, as a signal. The data clearly show that the entrapped liposomes are intact, as there is no response in the absence of ionomycin, and that the ionophore is membrane-associated, producing the desired fluorescence signal when Ca(II) is added.

Example 9: Liposome Microarrays using Transmembrane Ion Flux Signalling

An extension of the Fluo-3 based ion channel activity assay is the microarray format. Pin-printed sol-gel derived microarrays were constructed from samples illustrated in example 5. The microarrays were constructed with both negative and positive controls present. Negative controls consisted of buffered sodium silicate glass or fluo-3 loaded DOPC liposomes without ionomycin, while the positive control was entrapped fluorescein-dextran. In addition, the array contained fluo-3 loaded DOPC liposomes with ionomycin present within the membrane bilayer. It was clearly seen that upon addition of calcium ions to the exterior of the pin-printed array that only the samples containing the ionomycin ion channel underwent a change in fluorescence intensity, consistent with transmembrane ion flux and a corresponding increase in fluo-3 intensity (Figure 16). This example shows that the microarray format can be used to deposit intact liposome-ionophore assemblies onto surfaces, and to probe a functional response (i.e., transmembrane ion flux). Based on the other examples presented above, it is clear that such a microarray formation and readout method can be directly transferred to ligand gated ion channels such as the nicotinic acetylcholine receptor.

While the present invention has been described with reference to the above examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

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